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EVALUATION OF ANTI-OXIDANT AND ANTI-CANCER ACTIVITY OF ROOT EXTRACTS OF *ARTOCARPUS HIRSUTUS* BY IN-VITRO METHODS

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ABSTRACT

Artocarpus hirsutus is native to Kerala and Kanyakumari in India. It belongs to the family Moraceae. In conventional medicine, this species is found to be beneficial in treating acne, diarrhoea and ulcers. Here, we have selected this plant to evaluate antioxidant and anticancer activity. Anti-oxidant activity of Chloroform and ethanolic extracts of A. hirsutus root were studied by DPPH and ABTS method. The chloroform extract showed an IC50 value of 10, 3 µg/ml, and the IC50 value of standard ascorbic acid was 40 µg/ml. The ethanolic extract showed the IC50 value of 7, 1 µg/ml. Using extracts of ethanol and chloroform, the MTT assay was used to evaluate cytotoxic activity in the colon cancer cell line HT-29 and compared to the conventional drug 5-fluorouracil. The IC50 value of chloroform and ethanolic extract was found to be 58.71 µg/ml and 51.05 µg/ml respectively and the IC50 value of 5-fluorouracil is 8.8µg/ml. The ethanolic extract exhibited the greatest inhibition of the two extracts. Therefore, the ethanolic extract was tested for its ability to inhibit the normal Vero cell line and it was found to be less harmful. Further apoptotic investigation of the ethanolic extract was performed using DNA fragmentation and microscopic examination. After being treated with ethanolic extract, the cells revealed a reddish or orange colour and a loss of membrane integrity. This resulted in confirming that Artocarpus hirsutus ethanolic extract had an apoptotic effect on the colon cancerous cell line HT-29. The GC-MS analysis of the root extract of Artocarpus hirsutus identified compounds with drug-like properties based on ADME and toxicity prediction, indicating that this plant is a rich source of phytoconstituents with a high binding affinity in

docking experiments. Numerous noteworthy actions of the phytochemicals under investigation were uncovered by the phytoconstituents using the PASS analysis approach.

KEYWORDS: *Artocarpus hirsutus*, Pharmacology, PASS analysis, Anti-Cancer, Anti-oxidant, Toxicity prediction, HT-29, ADME properties, VERO cell line, GC-MS, *Artocarpus*, *Moraceae*.

INTRODUCTION

India contains abundant traditional medicinal plant resources with unique diversity. Using these medicinal plants to create new anticancer medications would be advantageous and represent a novel way of treating cancer.[1] *Artocarpus hirsutus*, frequently called Wild Jack, belongs to the Moraceae family. A. hirsutus is a tropical evergreen tree species, native to India (Karnataka, Kerala, Maharashtra, & Tamil Nadu). This enormous evergreen tree can grow to a height of seventy meters. Its leaves can grow up to 25x14cm and can be widely oval, oblong, or elliptic. Its bark is grey, and the hairs on its branches are tawny.

The seed oil of *A. hirsutus* is dark yellow in color and has a distinctly fragrant aroma. The seed powder has a dark brown to sandy appearance, and the seed kernel is bitter. A. hirsutus seed oil has the following specific activity values: 0.9471, acid value of 1.09, saponification value of 171.1, iodine value of 103.26, peroxide value of 6.76, and unsaponifiable matter of 5.83%.

Wild jack seed oil can cure wounds in both people and animals. The seeds are roasted by the tribe and consumed as snacks. This plant is used as a folk treatment for wounds, ulcers, joint pain, and other conditions. *A. hirsutus* fruits are used in traditional medicine to treat skin disorders such as acne, hydrocele, abscesses, and skin cracks. To treat diarrhea, soak the bark and roots of A. hirsutus for ulcers, apply the powdered bark topically. Leaves are used to treat hydrocele and buboes.[2]

They are widely acknowledged as a rich source of bioactive secondary metabolites such as flavonoids, stilbenes, triterpenoids, and xanthones. Secondary metabolites including stigmasterol, lupeol, cyclomorusin, cycloartomunin, and betulic acid are found in A. hirsutus roots.[3]

MATERIALS AND METHODS

Chemicals and drugs

Vero and HT-29 were obtained from the National Centre for Cell Sciences (NCCS), Pune, India. Cells were maintained in the logarithmic phase of growth in Dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, 1,1-diphenyl 2-picry hydrant, methanol, 2,20-azinobis-3- ethylbenzothiazoline-6-sulfonic acid, 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl- - tetrazolium bromide(MTT), 5-Fluorouracil, DMSO solution (0.1% v/v), Tryphan blue, Acridine orange/ Ethidium bromide (AO/EB), petroleum ether, chloroform and ethanol.

TE Buffer (10X; 100ml), Tris-HCl (0.1M) - 1.57 g, EDTA (0.01M; pH 8) - 292 g, SDS-10%, PCL solution (Phenol:Chloroform: Isoamyl alcohol), Sodium acetate, Isopropanol, Sterile distilled water.

Tools and database used

Pubchem, RCSPDB, molegro molecular viewer, Autodock 1.5.7, Swiss ADME, ProTox-II, PASS online tool.

Collection of plant material and authentication

The Root of Artocarpus hirsutus were collected from Marthandam, Kanyakumari district in the month of December and authenticated by Dr. D. Stephen Ph.D., Lecturer, Department of Botany, The American College, Madurai 02.

Preparation of Extract

The Plant Root was shade dried at room temperature and was subjected to size reduction to a coarse powder by using dry grinder. 120 grams of this coarse powder was packed into Soxhlet apparatus and was subjected to extraction sequentially with 750ml of petroleum ether, chloroform and ethanol. The extraction was continued until the colour of the solvent in the siphon tube became colourless. Extracts of chloroform and ethanol were subjected to evaporation by using Rotary evaporator at 60°C.

Culturing of cell lines

Vero (African green monkey kidney normal epithelial cell line) and HT-29 (Human colorectal adenocarcinoma epithelial cell line) were obtained from the National Centre for Cell Sciences (NCCS), Pune, India. Cells were maintained in the logarithmic phase of growth in Dulbecco's

modified eagle medium (DMEM) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin. They were maintained at 37°C with 5% CO2 in 95% air humidified incubator. The monolayer cells detached with trypsin-ethylene diamine tetra acetic acid (EDTA) to make single cell suspension and viable cells were counted using a hemocytometer and diluted with medium containing 10% FBS to give final density of 1×105 cells/ml. One hundred microlitres per well of cell suspension were seeded into 96- well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37ºc, 5% CO2, 95% air and 100% relative humidity. After 24 h the cells were treated with serial concentration of the samples. They were initially dissolved in neat dimethylsulfoxide (DMSO).

Percentage Yield

The percentage yield of both chloroform and ethanol extract was dried at room temperature and measured.

Qualitative phytochemical analysis

Chloroform and ethanol were tested for the presence of various active phytoconstituents namely steroids, alkaloids, tannins, phenolic compounds, flavonoids, diterpenes, triterpenes and saponins.[4]

EVALUATION OF ANTIOXIDANT ACTIVITY

DPPH scavenging activity

The DPPH scavenging activity of the sample was measured by 1,1-diphenyl,2-picry hydrant (DPPH) method Briefly, 0.4mM solution of DPPH in methanol was prepared and 2 ml of this solution was added to 5,10,20,40,60,80,100 (µg/ml) concentrations of both chloroform and ethanol extract and was allowed to stand at temperature for 20 mins, and then absorbance was read at 517 nm against blank samples. Reference compound being used was ascorbic acid and experiment was done on triplicate. Control cuvette contained all the reagents except the test sample extract.

Inhibition (%) = (Abs control - Abs sample / Abs control) x 100.[5]

ABTS radical scavenging activity

ABTS radical scavenging activity of the sample was measured by 2,20- azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) Briefly, ABTS solution (ABTS stock solution (7mM) and potassium persulfate solution (100mM)) in methanol was prepared and 2 mL of

this solution was added to 1, 2, 5, 10, 20, 40, 60, 80, 100 (µg/ml) concentrations of both chloroform and ethanol extract and was allowed to stand at room temperature for 20 mins and then absorbance was read at 734 nm against blank samples. Reference compound being used was ascorbic acid and experiment was done on triplicate. Control cuvette contained all the reagents except the test sample extract.

Inhibition (%) = (Abs control - Abs sample / Abs control) x 100[6]

Cytotoxic effect on Cell line

The cytotoxic effect of the sample was tested against HT-29 and vero cell lines by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Mossman, 1983). The cells were seeded in 96-well microplates (1 x 106 cells/well) and incubated at 37°C for 48 h in 5% CO2 incubator and allowed to grow 70-80% confluence. Then the medium was replaced and the cells were treated with 20,40,60,80,100 (µg/ml) concentrations of Chloroform and ethanol extract and incubated for 24 h. The morphological changes of untreated (control) and the treated cells were observed under digital inverted microscope (20X magnification) after 24 h and photographed. The cells were then washed with phosphate-buffer saline (PBS, pH-7.4) and 20 µL of (MTT) solution (5 mg/mL in PBS) was added to each well. The plates were then stand at 37ºC in the dark for 2 h. The formazan crystals were dissolved in 100 µL DMSO and the absorbance was read spectrometrically at 570 nm.

Cell viability (%) = (Absorbance of sample/Absorbance of control) X 100[7]

Fluorescence Microscopy Studies

Acridine Orange (AO)/Ethidium Bromide (EB)

Drug-mediated apoptosis-associated changes in HT-29 cells were investigated by Acridine orange/ Ethidium bromide (AO/EB) dual staining Both viable and nonviable cells absorb AO stain and emit green fluorescence, whereas EO stain has been absorbed only by nonviable cells and emits red fluorescence by losing their membrane integrity. The HT-29 cells were cultured in a 6-well plate (1 × 103 cells per well) and treated with 100µg/ml concentration of ethanol extract for 24 h. The untreated HT-29 cells served as control. The treated cells were washed with PBS and stained with 20 µl of AO/EB staining (100 µg/ml AO and 100 µg/ml EB) solution for 5 min. The stained cells were viewed under a fluorescence microscope (Invitrogen EVOS FL Cell Imaging; 40× magnification).[8]

DNA Fragmentation study

The sample which possesses best anti-cancer activity will be used for the DNA fragmentation study. Transfer treated cells to tube and pellet the cells through centrifugation at 2000 rpm for 5 minutes. Discard the supernatant. Resuspend pellet with 600µL. TE Buffer and 200 µL lysis buffer and gently mix and incubate at 37°C for 30 minutes. Add 30 µL 10% SDS (sodium dodecyl sulfate) and 3µL proteinase K, gently invert and incubate at 50°C for 15 minutes. Add the equal volume of PCI (Phenol: Chloroform:Isoamyl) solution and mix for 10 minutes by gentle inversion. Centrifuge at 8.000 rpm for 10 minutes. Extreme care and personal protective gear (gloves, lab coats, and safety goggles) should be used when working with phenol as it is corrosive and may cause severe burns. This step should be completed in a fume hood. Transfer the upper aqueous phase to a sterile 1.5-mL microcentrifuge tube, taking care not to disturb the bilayer. Sodium acetate (5M, 100 µL) is added to the contents and is mixed gently. Equal volume of isopropanol is added and mixed gently by inversion till white strands of DNA precipitates out. The contents are centrifuged at 5,000 rpm for 10 minutes. Add 500 µl of (-20°C) 100% ice cold ethanol and gently mix by inversion. Centrifuge at 8,000 rpm for 10 minutes. Carefully decant the supernatant and thoroughly dry pellet at room temperature or in a 37°C incubator. Over drying will result in making the DNA pellet more difficult to dissolve back into solution. The pellet may or may not be visible to the naked eye. Resuspend the pellet in 25-50 µL TE (Tris-EDTA) buffer and allow pellet to sit overnight at 4°C. Confirm presence and concentration of bacterial DNA by running 5 µL of product on a 1% agarose gel, Purified DNA will appear as a defined band width.[9]

GC MS Analysis

GC-MS analysis was performed with Agilent GC 7890A 240MS with Ion Trap gas-chromatograph equipped with (HP5, 30 meters, 0.32mm x 0.25µm) HP5 capillary column (30mx0.32 mm; coating thickness 0.25 µm) interfaced with Agilent 240 MS Ion Trap mass detector. Analytical conditions: Injector and transfer line temperature 220 and 2400oC, respectively oven temperature programmed from 800 ^{o}C to 3000 ^{o}C at 40C/min; carrier gas, helium at 1 ml/min; injection 0.2 µl of a n- hexane. The identification of the components was performed for both the columns by comparison of their retention times with those of pure, authentic samples and by mean of their liner retention indices and by computer matching against commercial mass spectra libraries NIST and MS literature data.[10]

ADME Study

The online web tool Swiss ADME was used to evaluate the ADME parameters of the all GC-MS compounds using Lipinski's rule of five.[11] Lipinski stated that a compound could display drug-like behavior if it does not fail more than one of the criteria such as; (i) MW not more than 500; (ii) Hydrogen bond donors ≤ 5; (iii) Hydrogen bond acceptors ≤ 10; (iv) Lipophilicity < 5; and (v) molar refractivity between 40 and 130. Those compounds are considered ideal drug candidates which obey the Lipinski rule.[12]

Molecular Docking

Preparation of ligand and protein.

2D structure of "Phytoconstituents identified by GC-MS" was downloaded from the PubChem website. https://pubchem.ncbi.nlm.nih.gov/

3D structure of the protein was downloaded from Pdb (Protein Data Bank) website.

https://www.rcsb.org.

METHODOLOGY

The three-dimensional crystal structure of p53 CO-crystallized structure of the human p53 core domain mutant M133L/V203A/N239Y/N268D (PDB ID:1UOL, at 1.90 A resolution) was retrieved from the protein data bank. Molegro molecular viewer was used to remove the ligand and water molecule from the protein and help us get the exact protein structure alone. Autodock software was used to read the molecule, adding the polar hydrogens and Kollman charges. Later, Grid box was created, and set appropriately to produce a perfect grid box. Once protein and ligand were docked, torsion was selected, and the output file was selected in PDBQT format in MGL tools. The output was visualized using molegro molecular viewer software, and validation was evaluated.[13]

In Silico Toxicity Prediction Study

Toxicity and Lethal Dose (LD50) predictions for identified compounds were carried out by using ProTox-II. Accessed on (7 feb 2024).

In Silico PASS Prediction Study

The Prediction of Activity Spectra for Substances (PASS) online tool was utilized to assess the potential bioactivities of the best-docked compounds. This program can predict up to 3750 bioactivities of a molecule based on a chemical structural analysis.[14]

RESULTS

Percentage yield

The percentage yield of extract of *Artocarpus hirsutus* was shown in Table No.1, All the extracts were stored in a well-closed container at 4^0C.

Table 1: Percentage yield of extract of *Artocarpus hirsutus*.

Extract	Percentage yield(%w/w)
Chloroform	6.26
Ethanol	4.75

Qualitative phytochemical analysis

Phytochemical analysis was carried out for chloroform and ethanol extract of root of *Artocarpus hirsutus* and the results are shown in Table No.2.

Table 7: Result of Phytochemical analysis of *Artocarpus hirsutus* extract of chloroform and ethanol.

Constituents	Chloroform extract	Ethanol extract
Alkaloids	Present	Present
Carbohydrate	Present	Present
Glycosides	Absent	Present
Saponins	Present	Absent
Protein and Aminoacids	Absent	Absent
Aminoacids	Absent	Absent
Phenolic compound	Present	Present
Flavanoids	Present	Present
Terpenoids	Present	Present
Steroids	Absent	Present

The phytoconstituents present in chloroform extract such as Alkaloids, Carbohydrates, Saponins, Phenolic compounds, Flavonoids and Terpenoids. Ethanolic extract showed the presence of Alkaloids, Carbohydrates, Glycosides, Phenolic compounds, Flavonoids, Terpenoids, and Steroids.

DPPH radical scavenging assay

The free radical scavenging activity of root extracts *Artocarpus hirsutus* was carried out by DPPH method. The ethanolic and chloroform extract was evaluated and compared with standard Ascorbic acid. The chloroform extract shows IC50 value of 10µg/mL and ethanolic extract shows IC50 value of 7(µg/mL) and IC50 value of standard ascorbic acid is 40 µg/ml.

The chloroform and ethanolic extracts have higher inhibition at low concentration when compared with standard ascorbic acid.

ABTS scavenging assay

The free radical scavenging activity of root extracts *Artocarpus hirsutus* was carried out by ABTS scavenging assay. The ethanolic and chloroform extract was evaluated and compared with standard Ascorbic acid. The chloroform extract shows IC50 value of 3µg/ml and ethanolic extract shows IC50 value of 1µg/ml and IC50 value of standard ascorbic acid is 40 µg/ml. The chloroform and ethanolic extracts have higher inhibition at low concentration when compared with standard ascorbic acid.

Cytotoxicity studies: MTT assay

The cytotoxic activity of chloroform and ethanolic extracts were evaluated on colon cancer cell line HT-29 and compared with standard drug 5-fluorouracil. The IC50 value of ethanolic and chloroform extract is 51.05µg/ml and 58.71 µg/ml respectively. The IC50 value of standard 5-Fluorouracil was found to be 8.8µg/ml. which is given in table no.3,4

Table No 3: IC50 concentration and %cell viability of chloroform and ethanolic extract of *Artocarpus hirsutus* in HT-29 Cell line.

	Concentrations (µg/ml)	C	20	40	60	80	100	IC50 Value
Chloroform extract	**Cell Viability (%)**	100	89.92± 0.003	64.18± 0.002	41.67± 0.003	30.21± 0.002	18.30± 0.002	**58.71 (µg/ml)**
Ethanol extract		100	85.34± 0.003	60.04± 0.005	33.06± 0.006	16.66± 0.001	12.71± 0.003	**51.05 (µg/ml)**

Table No 4: IC50 concentration and % cell viability of 5-Fluorouracil in HT-29 Cell line.

	Concentrations (µg/ml)	C	5	10	15	20	25	IC50 Value
5-flurouracil	**Cell Viability (%)**	100	84.05± 0.003	45.09± 0.005	31.57± 0.006	12.77± 0.001	8.68± 0.003	**8.8 (µg/ml)**

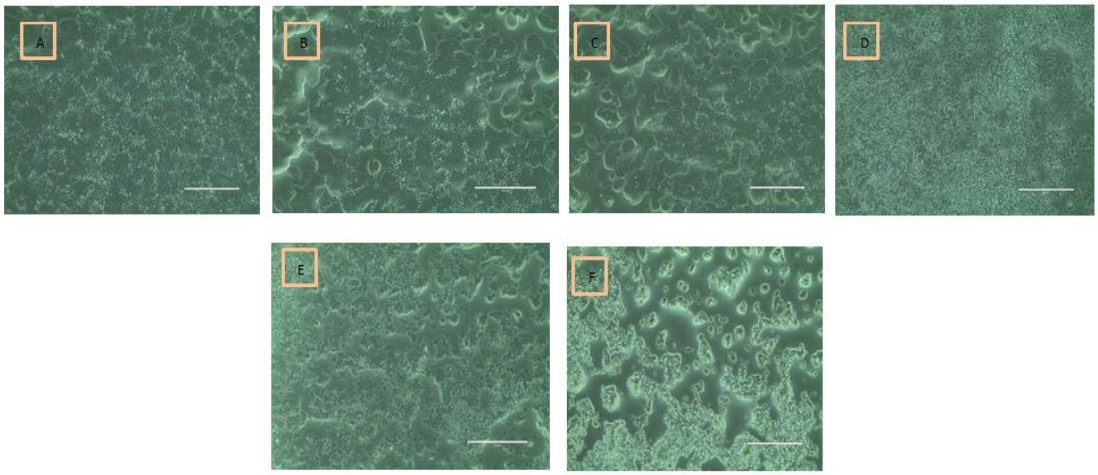

Figure 1: HT-29 cells treated with chloroform extracts-(A) Control, (B) 20 µg/ml, (C) 40 µg/ml, (D) 60 µg/ml, (E) 80 µg/ml, (F) 100 µg/ml.

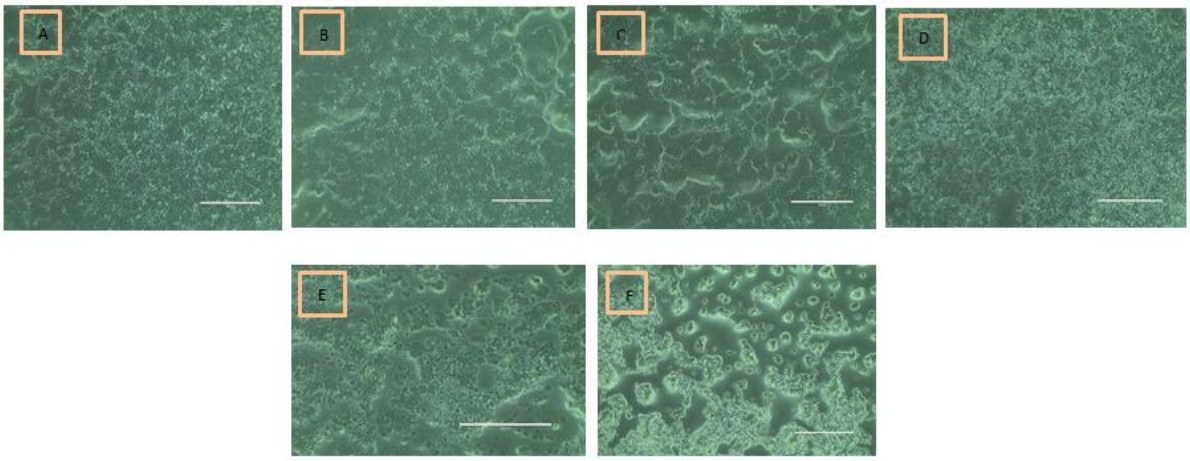

Figure 2: HT-29 cells treated with Ethanolic extracts – (A) Control, (B) 20 µg/ml, (C) 40 µg/ml, (D) 60 µg/ml, (E) 80 µg/ml, (F) 100 µg/ml.

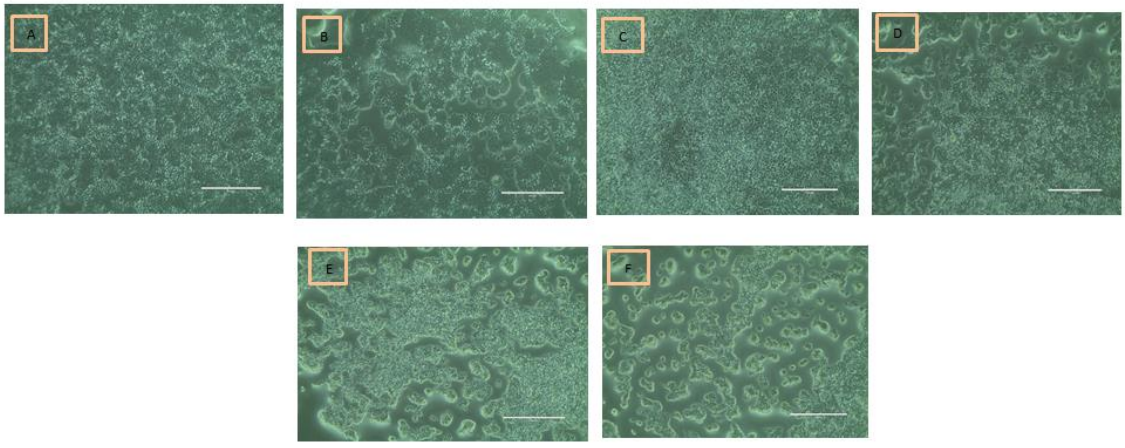

Figure 3: HT-29 cells treated with 5-fluorouracil – (A) Control, (B) 5 µg/ml, (C) 10 µg/ml, (D) 15 µg/ml, (E) 20 µg/ml, (F) 25 µg/ml.

Using MTT assay, it was found that the ethanolic extract was more effective than chloroform extract. So, the ethanolic extract was selected to assess the cytotoxicity against the normal Vero cell line and the results should that it is less harmful against the Vero cell line. which is given in table no.5. Thus, the ethanolic extract inhibits the growth and more toxic against the cancer cell line as compared to Vero cell line.

Table no. 5: % cell viability of ethanolic extracts of *Artocarpus hirsutus* in Vero cell line.

	Concentrations (µg/ml)	**C**	**20**	**40**	**65**	**80**	**100**
Ethanolic extract	**Cell Viability (%)**	100	98.85±0.002	95.81 ±0.003	93.91±0.003	89.86±0.004	87.07±0.002

The MTT assay were conducted on VERO Cell line using ethanolic crude extract of *Artocarpus hirsutus* and image are given in figure 4.

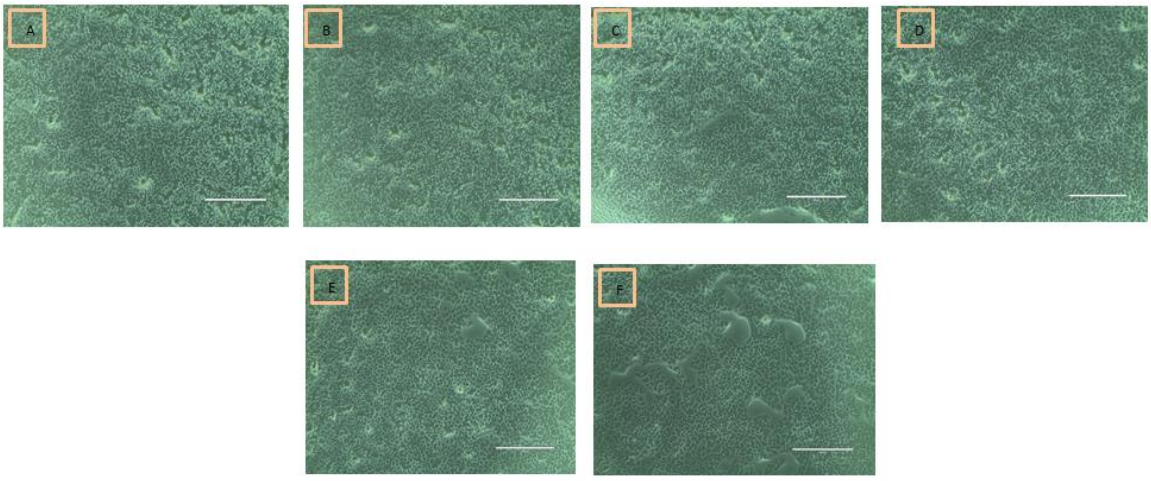

Figure 4: VERO cells treated with Ethanolic extracts – (A) Control, (B) 20 µg/ml, (C) 40 µg/ml, (D) 60 µg/ml, (E) 80 µg/ml, (F) 100 µg/ml.

Flourescence microscopic observation

Control cells without any drug or extract treated shows bright green colour, which indicates the viable cells. Cells treated with Ethanolic extract of *Artocarpus hirsutus* shows bright orange colour with loss of membrane integrity and red colour indicates the dead cells.

The observations of fluorescent microscopic studies are depicted in figures 5 & 6.

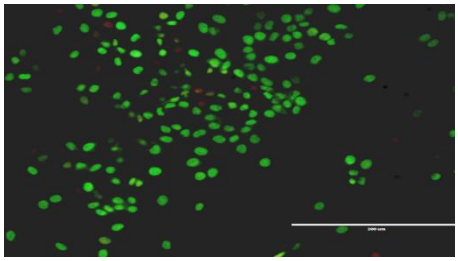

Figure 5

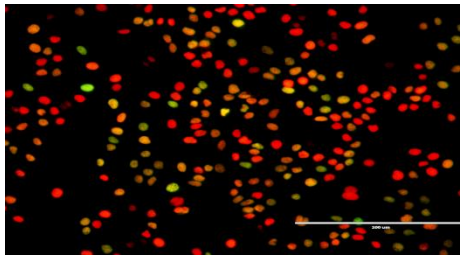

Figure 6

Figure 5: HT-29 cells – Control, indicting viable cells stained green in colour and

Figure 6: HT-29 cells after treatment with Ethanolic extract of *Artocarpus hirsutus* showing dead cells stained orange and red in colour with loss of membrane integrity.

DNA Fragmentation observation

In the control HT-29 cells, there was no fragmentation observed in agarose gel.

Fragmentation was observed in HT-29 cell treated with IC50 concentration of ethanolic extract of root of *Artocarpus hirsutus*.

Results obtained in fragmentation studies are shown in figure no:7

L C T

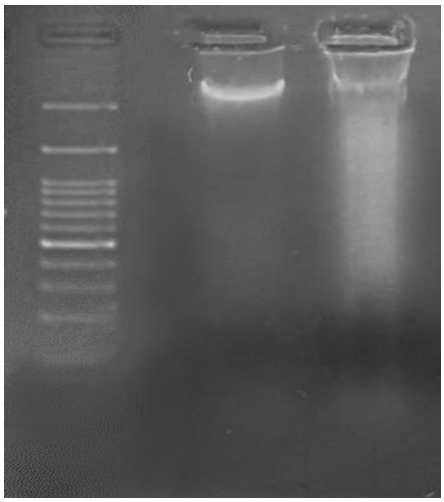

Figure 7: Results obtained in fragmentation studies.

L: 100 base pair DNA marker; **C**: HT-29 cells without any treatment.

T: HT-29 cells treated with ethanol extract of *Artocarpus hirsutus*.

GC-MS Analysis

GC-MS analysis revealed that the presence of bioactive compounds. The ethanolic extract shows the presence of Lanosta-8,24-dien-3-one in 4.13, .alpha.-Amyrin in 4.57, 1,7-Di(dodec-9-ynyl)-2,2,4,4,6,6-hexamethyl-1,3,5,7-tetraoxa-2,4,6-trisilahep in3.29, Ergosterol

peroxide Me derivative in 6.40, Masticadienonic acid , methyl ester (isomer 1) in 3.11, (3R,5aR,9S,9aS)-2,2,5a,9-Tetramethyloctahydro-2H-3,9a-methanobenzo[b]o in 3.99, 9,19-Cyclolanostan-3-ol, 24-methylene- in 7.17, (3.beta.)-, Lup-20(29)-en-3-one in 8.04, 3-Dodecyloxypropanoic acid, TMS 2.63, Lanosta-8,24-dien-3-ol, acetate, (3.beta.)- in peak of 4.54.

Table no. 6: GC-MS Analysis of ethanolic extract of *Artocarpus hirsutus.*

Peak#	R.Time	Area	Area%	Height	Height%	A/H	Name
1	26.868	208009	8.42	50340	10.45	4.13	Lanosta-8,24-dien-3-one
2	27.905	124081	5.02	27128	5.63	4.57	.alpha.-Amyrin
3	28.014	142709	5.78	43389	9.01	3.29	1,7-Di(dodec-9-ynyl)-2,2,4,4,6,6-hexamethyl-1,3,5,7-tetraoxa-2,4,6-trisilahep
4	28.880	211112	8.55	32994	6.85	6.40	Ergosterol peroxide Me derivative
5	28.964	124322	5.03	39965	8.30	3.11	Masticadienonic acid, methyl ester (isomer 1)
6	29.915	189393	7.67	47504	9.86	3.99	(3R,5aR,9S,9aS)-2,2,5a,9-Tetramethyloctahydro-2H-3,9a-methanobenzo[b]oxepine
7	30.013	626215	25.36	87296	18.13	7.17	9,19-Cyclolanostan-3-ol, 24-methylene-, (3.beta.)-
8	30.185	577715	23.39	71889	14.93	8.04	Lup-20(29)-en-3-one
9	30.230	140207	5.68	53307	11.07	2.63	3-Dodecyloxypropanoic acid, TMS
10	30.773	125852	5.10	27744	5.76	4.54	Lanosta-8,24-dien-3-ol, acetate, (3.beta.)-
		2469615	100.00	481556	100.00		

ADME property prediction for the GC-MS compounds.

The ADME properties of GC-MS compounds were studied using the online program SwissADME and ADMEcalculator to further investigate their pharmacokinetics, drug-likeness, physiochemical properties. According to Lipinski's rule of five, 3R,5aR,9S,9aS)-2,2,5a,9-Tetramethyloctahydro-2H-3,9a-methanobenzo[b]oxepine, 3-Dodecyloxypropanoic acid are pass the all rule of five.

Ergosterol peroxide, alpha-Amyrin and Lupenone are violation in Liphophilicity and the Masticadienonic acid is violation in Molar refractivity.

When a drug violates 2 or more of these conditions considered a non-orally available drug. Except 1,7-Di(dodec-9-ynyl)-2,2,4,4,6,6-hexamethyl-1,3,5,7-tetraoxa-2,4,6-trisilahep, Lanosterone, 24-Methylenecycloartanol, Lanosteryl acetate are have 0 to 1 violation.

According to Lipinski's rule almost all the GC-MS compounds showed orally active drug-likeness properties. It is reported that compounds with lower lipophilicity, molecular weight, and hydrogen bond capacity have high permeability, good absorption, and bioavailability.

Result of Docking study

The results are summarized. The results revealed that the p53 regulatory property of the compounds from the GC-MS report of *Artocarpus hirsutus* greatly depends on the chemical nature of the substituents. The docking studies of the ligands to protein active sites were performed by an autodock software 1.5.7 for determining the binding affinities of the compounds. The designed analogs were docked toward the p53 (PDB id: 1UOL) to ascertain their stabilization activity.

The some of the chemical constituents from *Artocarpus hirsutus* such as are alpha-Amyrin, Ergosterol peroxide, Lupenone significantly active with p53 with significant binding energy more when compared to currently used drug 5-fluorouracil (-6.8).

The above compounds have good affinity to the receptor due to more lipophilic character and also due to hydrogen bonding. The results are summarized. The best‑affinity modes of all the docked compounds with p53 (PDB id:1UOL) are shown. Almost all the compounds are docked in the same binding pocket. The docking results of the compound *Artocarpus hirsutus* exhibited interactions with p53 and the residues ASP148, GLN144, THR102, ASN131, TYR126, PHE113, SER269, THR126, HIS178, MET243,. Plays a crucial role in binding with ligands.

The Ergosterol peroxide have a highest binding energy (-7.59) of all compounds and also higher than standard.

Table no 7: Report of molecular docking of p53 protein.

Compound name	Binding energy	Hydrogen bond interaction	Steric interaction
Lanosterone	-6.55	ASP148	ASP148, ASP228, VAL147 PRO223
alpha-Amyrin	-7.05	GLN144	TRP149, LEU111, TYR126
Ergosterol peroxide	-7.59	THR102ASN131 TYR126	ASP26, GLY112, LEU111
Masticadienonic acid	-5.75	PHE113	TYR126, PHE133, LEU111 ASP268
Froggatt ether	-4.93	----------	MET160, PRO98

24-Methylenecycloartanol	-6.04	SER269	LEU130, ASN131, LEU252 GLU271
Lupenone	-7.17	----------	GLY112, LEU111, ASP268 TYR126, ASN131
3-Dodecyloxypropanoic acid	-2.17	TYR126	LEU111, PHE113
Lanosterol acetate	-6.27	HIS178, MET243	HIS178, MET243
5-FLUOROURACIL	-6.8	ALA159,HIS214 LEU194	ALA161, VAL216 1LE195

Toxicity Prediction class of best docked compounds by ProTox-II.

S.no	Compound	Predicted LD50, (mg/kg)	Predicted Toxicity Class
1.	Lanosterone	5000	5
2.	alpha-Amyrin	70000	6
3.	Ergosterol peroxide	2340	5
4.	24-Methylenecycloartanol	5000	5
5.	Lupenone	5000	5
6.	Lanosteryl acetate	4300	5
SD	5-FLUOROURACIL	1190	4

ProTox (http://tox.charite.de/protox_II, accessed on 1 feb 2024) Class 1: deadly if consumed (LD50 ≤ 5); Class 2: deadly if consumed (5 < LD50 ≤ 50); Class 3: lethal if consumed (50 < LD50 ≤ 300); Class 4: harmful if consumed (300 < LD50 ≤ 2000); Class 5: maybe harmful if consumed (2000 < LD50 ≤ 5000); Class 6: non-lethal (LD50 > 5000).

Biological Activity Prediction

The web tool for predicting bioactivity based on structure the potential biological activity of the best docked compounds has been evaluated using Prediction of Activity Spectra for Substances (PASS). The biological activity and potential targets of each chemical were identified by the PASS research. We examined the biological activity of every molecule by looking at Pa > Pi and Pa > 7. The findings indicated a number of significant activities with Pa > 0.7, indicating this species' greater potential.

All the compound which show anti-cancer related activity such as Apoptosis agonist, Caspase 3 stimulant, Chemopreventive, Antineoplastic.

DISCUSSION

Globally, Cancer is considered as a major health issue. Globally, Colorectal cancer is in the second highest rate of cancer-related mortality. It was estimated that in 2020, there were over 1.9 million newly diagnosed cases of colorectal cancer and over 930,000 deaths from the disease.

It is necessary to find novel approaches to cancer care because the mortality rates for cancer patients are rising and clinical interventions including radiation, chemotherapy, immune modulation and surgery are not always successful in treating cancer patients.

Phytochemicals from medicinal plants are increasingly recognized for their ability to treat various disorders, including cancer. Several studies have evaluated plant extracts as potential preventive medicines to prevent carcinogenesis.

Approximately 60% of authorized cancer treatments come from natural sources. Following extensive folk practices, several Indian remedies have been evaluated and utilized for the treatment and prevention of a variety of chronic conditions, including cardiovascular disease and cancer.

Natural phytochemicals can inhibit tumour progression through various mechanisms, including genotoxic effects, anti-inflammatory, anti-oxidant effects, cell proliferation inhibition, and protection of intracellular communication to modulate apoptosis and signal transduction pathways.

Screening plant components for anti-cancer medicines is a major scientific activity globally. In the 1950s, vinca alkaloids and cytotoxic podophyllotoxins were identified as the first plant-derived anti-cancer medicines. Several natural chemicals, including camptothecin, vincristine, vinblastine, taxol and podophyllotoxin have been structurally changed to create stronger anti-cancer drugs with fewer side effects.

Artocarpus hirsutus plant used to treat wounds, ulcers, joint pain and other ailments. In traditional medicine, *A. hirsutus* fruits are used to treat skin conditions like acne, hydrocele, abscesses, and cracks in the skin. *A. hirsutus* bark and roots can be steeped to treat diarrhoea and the powdered bark can be applied topically to treat sores.

Phytochemical study showed the presence of Alkaloids, Carbohydrates, Saponins, Phenolic compounds, Flavonoids, Terpenoids, Glycosides and Steroids.

It has been reported with anti-oxidant, anti-bacterial, anti-fungal, anti-diabetic, anti-arthritic, anti-inflammatory and analgesic activity. There was no previous study to prove its *Artocarpous hirsutus* root contain anti-cancer activity. So, the study was carried out to evaluate the anti-cancer effect of root extract of *Artocarpus hirsutus.*

Dried Root of *Artocarpus hirsutus* was extracted with solvents like petroleum ether, chloroform and Ethanol. Petroleum ether solvent is used to remove fats and oils from the root.

The anti-oxidant activity of root extracts *Artocarpus hirsutus* was carried out by two methods such as DPPH and ABTS radical scavenging assay. The chloroform extract showed the IC50 value of 10, 3 µg/ml and the IC50 value of standard ascorbic acid showed 40 µg/ml.

The ethanolic extract showed the IC50 value of 7, 3 µg/mL and the IC50 value of standard ascorbic acid 40 µg/ml.

Cytotoxic activity was carried out in colon cancerous cell line HT-29 with extracts of chloroform, ethanol and was compared with standard drug 5-Fluorouracil.

Test for cytotoxicity was carried out by MTT assay for both chloroform and ethanolic extract. The IC50 value of 51.05 µg/ml and chloroform extract with IC50 value of 58.71 µg/ml. And the IC50 value of 5-Fluorouracil was found to be 8.8µg/ml.

Among the two extracts, Ethanolic extract showed highest inhibition. Hence, the ethanolic extract was evaluated for inhibition of normal Vero cell line and it should be founded as less toxicity again the Vero cell line.

The ethanolic extract was further subjected to apoptotic study by Microscopic analysis and DNA fragmentation.

Apoptotic effect of ethanolic extract of *Artocarpus hirsutus* treated HT-29 cells was confirmed with the help of fluorescence microscopy using acridine orange and ethidium bromide. Acridine orange is a vital dye capable of staining both dead and live cells, whereas ethidium bromide will stain only cells that have lost their membrane integrity. On examination, the control cells i.e., without any treatment under fluorescent microscope, the cells were stained green in colour representing viable or live cells, whereas examination of cells after treatment with ethanolic extract showed reddish or orange colour with loss of membrane integrity. This led to confirmation that ethanolic extract of *Artocarpus hirsutus* showed apoptotic effect in colon cancerous cell line HT-29.

DNA fragmentation study was carried out by extracting DNA from the cells after treatment with IC50 concentration of ethanolic extract of *Artocarpus hirsutus* for 48 hours and also from cells without any treatment. Apoptosis is characterized by cleavage of chromosomal DNA into oligonucleosomal fragments. Cleavage of chromosomal DNA into oligonucleosomal fragments is a hallmark of apoptosis and apoptotic cells often produce nucleotide fragments and visualized by DNA agarose gel electrophoresis. On examination, DNA fragmentation appeared in HT-29 cells treated with ethanolic extract and compared to cells without any treatment, which did not show any fragmentation. This fragmentation of DNA indicated the characteristics of apoptotic cells. Thus, ethanolic extract of *Artocarpus hirsutus* causes DNA damage in HT-29 cells, thereby inducing apoptosis.

The GC-MS analysis is carried out for ethanolic extract of *Artocarpus hirsutus* which show the presence of are Lanosta-8,24-dien-3-one in 4.13,. alpha.-Amyrin in 4.57, 1,7-Di(dodec-9-ynyl)-2,2,4,4,6,6-hexamethyl-1,3,5,7-tetraoxa-2,4,6-trisilahep in 3.29, Ergosterol peroxide in 6.40, Masticadienonic acid, methyl ester (isomer 1) in 3.11, Froggatt ether in 3.99, 9,19-Cyclolanostan-3-ol, 24-methylene- in 7.17, (3.beta.)-, Lup-20(29)-en-3-one in 8.04, 3-Dodecyloxypropanoic acid, TMS 2.63, Lanosta-8,24-dien-3-ol, acetate, (3.beta.)- in peak of 4.54.

The in-silico study was carried out for GC-MS compound. The ADME properties of GC-MS compounds were studied using the online program Swiss ADME to further investigate their pharmacokinetics, drug-likeness, physiochemical properties. According to Lipinski's rule of five, Froggatt ether, 3-Dodecyloxypropanoic acid are pass the all rule of five.

Ergosterol peroxide, alpha-amyrin and Lupenone are violation in Liphophilicity and the Masticadienonic acid is violation in Molar refractivity.

The compounds which pass the ADME properties were docked with the p53 protein (PDB ID:1UOL) and the binding efficiency of the compounds were studied. All the compounds had binding scores that were between -2.17 and -7.59 kcal/mol and three compounds are better than the standard drug. The Ergosterol peroxide compound has higher binding energy when compared with other compounds. A negative score in the scoring represents the minimum binding energy required to form a complex between a protein complex and a ligand.

Toxicity Prediction class of best docked compounds by ProTox-II. alpha -Amyrin is predicted as Class 6 as non-lethal. And all other compound is predicted as class 5. In this study, all the compounds showed prediction for low toxicity.

The potential biological activity of the best docked compounds has been evaluated using Prediction of Activity Spectra for Substances (PASS). The biological activity and potential targets of each chemical were identified by the PASS research.

All the compound which show anti-cancer related activity such as Apoptosis agonist, Caspase 3 stimulant, Chemopreventive, Antineoplastic.

The Ergosterol peroxide compounds have passed the ADME test and which has higher binding energy when compared with standard. At the same time which is toxicity predicted as the class 5. And in pass analysis Ergosterol peroxide show significant anticancer related activity.

In previous literature Ergosterol peroxide had cytotoxic effects on HT29 cells and elevated ROS levels. Ergosterol peroxide increased the expression of oxidative stress-inducible genes and the cyclin-dependent kinase inhibitor CDKN1A, while suppressing STAT1 and interferon-inducible genes.

Ergosterol peroxide inhibited proliferation of cells and clonogenic colony formation in HCT116, HT-29, SW620, and DLD-1 CRC cell lines. The growth inhibition found in these CRC cell lines was due to apoptosis, as validated by FACS analysis and Western blotting. Ergosterol peroxide reduced the nuclear levels of β-catenin.

SUMMARY AND CONCLUSION

The percentage yield of ethanolic and chloroform extract was found to be 4.75 and 6.26 respectively.

Evaluation of Phytochemical analysis of Chloroform and ethanolic root extracts of *Artocarpus hirsutus* revealed the presence of Alkaloids, Carbohydrates, Saponins, Phenolic compounds, Flavonoids, Terpenoid, Glycosides, steroids.

The anti-oxidant activity of *Artocarpus hirsutus* root has been screened using DPPH, ABTS method and compared with standard ascorbic acid and the both extracts showed higher anti-oxidant activity than the standard.

In this present study, Ethanolic extract of *Artocarpus hirsutus* was found to possess potent cytotoxic activity in human colon cancerous cell line HT-29 and was compared with standard drug 5- Fluorouracil.

At the same time the ethanolic extract was further subjected to test cytotoxicity against Vero cell line and showed less toxic against the normal Vero cell line. By this study, it is confirmed that the ethanolic extract produce less toxicity on non-cancerous cells.

The Apoptotic effect was confirmed in ethanolic extract treated cells by appearance of loss of membrane integrity and fragmentation of DNA.

The GC-MS study of *Artocarpus hirsutus* root extract demonstrated that this plant is a rich source of phytoconstituents with a high binding affinity in docking studies and revealed drug-like characteristics compounds based on ADME and toxicity prediction. The PASS analysis approach for phytoconstituents revealed numerous significant actions of the phytochemicals investigated.

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Conflict of Interest

The authors declare no conflict of interest.

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